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Characteristics and Osmoregulatory Roles of Uptake Systems for Proline and Glycine Betaine in *Lactococcus lactis*

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Lactococcus lactis subsp. *lactis* ML3 contains high pools of proline or betaine when grown under conditions of high osmotic strength. These pools are created by specific transport systems. A high-affinity uptake system for glycine betaine (betaine) with a K_m of 1.5 μ M is expressed constitutively. The activity of this system is not stimulated by high osmolarities of the growth or assay medium but varies strongly with the medium pH. A low-affinity proline uptake system (K_m , >5 mM) is expressed at high levels only in chemically defined medium (CDM) with high osmolarity. This transport system is also stimulated by high osmolarity. The expression of this proline uptake system is repressed in rich broth with low or high osmolarity and in CDM with low osmolarity. The accumulated proline can be exchanged for betaine. Proline uptake is also effectively inhibited by betaine (K_i of between 50 and 100 μ M). The proline transport system therefore probably also transports betaine. The inhibition of proline transport by betaine results in low proline pools in cells grown in high-osmotic-strength, betaine-containing CDM. The energy and pH dependency and the influence of ionophores on the activity of both transport systems suggest that these systems are not proton motive force driven. At low osmolarities, proline uptake is low but significant. This low proline uptake is also inhibited by betaine, although to a lesser extent than in cells grown in high-osmotic-strength CDM. These data indicate that proline uptake in *L. lactis* is enzyme mediated and is not dependent on passive diffusion, as was previously believed.

Glycine betaine (*N,N,N*-trimethylglycine) or betaine and proline are well-known osmoprotectants and compatible solutes of bacteria (6, 7). At high medium osmolarity, these compounds have a growth-stimulating effect in many bacteria. They are accumulated to very high intracellular concentrations without apparent deleterious effects on cell physiology. Most bacteria do not synthesize betaine de novo and are dependent on uptake of betaine or its precursor, choline. A natural source of betaine may be found in degrading plant and animal material. Uptake of betaine has been studied in a number of bacteria. In bacteria in which the regulation of transport was studied, the transport systems were found to be induced or activated by high osmolarity of the medium (1, 9, 10, 17). Proline is also an osmoprotectant in bacteria which possess an osmotically induced or activated proline transport system (2, 11, 14, 24). Some bacteria under osmotic stress increase the proline pool of endogenous origin by regulation of proline metabolism (12).

Osmoregulation in *Lactococcus lactis* subsp. *lactis*, an important organism in starter cultures used in the dairy industry, has not been described before. Such a study is of importance when conditions which favor survival under conditions of low water activity (osmotic stress), for example, in the freeze-dried state, are sought.

MATERIALS AND METHODS

Media and growth conditions. *L. lactis* subsp. *lactis* was grown in modified MRS broth (8). This contained (in grams per liter, if not stated otherwise) tryptone (Difco, Detroit, Mich.), 10; Lab-Lemco powder (Oxoid, Basingstoke, England), 10; yeast extract, 5; K_2HPO_4 , 2; $MgSO_4 \cdot 7H_2O$, 0.2;

$MnSO_4 \cdot H_2O$, 0.036; and 0.5 ml of Tween 80 (Merck, Munich, Germany) per liter. The pH was adjusted with HCl to 6.3. After sterilization, separately sterilized lactose was added to a concentration of 20 g/liter.

The chemically defined medium (CDM) also used for growth of *L. lactis* subsp. *lactis* has been described elsewhere (16) and was modified as follows. Instead of casein hydrolysate, it contained the following amino acids (in milligrams per liter): alanine, 237.5; glutamine, 390; asparagine $\cdot H_2O$, 350; arginine, 125; lysine $\cdot HCl$, 437.5; isoleucine, 212.5; methionine, 125; phenylalanine, 275; serine, 337.5; threonine, 225; tryptophan, 50; valine, 325; glycine, 175; histidine $\cdot HCl \cdot H_2O$, 150; leucine, 475; proline, 675; and tyrosine, 290.

The incubation temperature for growth in all experiments was 29°C.

Synthesis of radiolabeled betaine from radiolabeled choline. Radiolabeled betaine was made from 3H -labeled (78.2 kCi/mol) or ^{14}C -labeled (55 Ci/mol) choline (Amersham, Buckinghamshire, Great Britain) by the method described by Landfald and Ström (13). Purity was checked by thin-layer chromatography as described by Speed and Richardson (23). This showed that all choline was converted to a product that comigrated with nonradiolabeled genuine betaine. In some experiments, ^{14}C -labeled betaine, produced on request by Amersham, was used (55 Ci/mol).

Extraction of cells to determine intracellular radiolabeled betaine and proline concentrations. A 10-ml culture was grown in the presence of ^{14}C -betaine or ^{14}C -proline. While the culture was still in the exponential growth phase, its optical density at 660 nm was determined, and the protein content (in milligrams per milliliter) was deduced from this value by multiplying by a factor of 0.2 (the ratio between the protein concentration [as determined by the method of Lowry et al.] and the optical density is fairly constant for suspensions of washed cells grown under different condi-

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tions). The culture was then filtered over a cellulose-nitrate filter (0.45- μ m pore size; BA85; Schleicher & Schuell, Dassel, Germany). The filter was transferred to a glass vial, and 0.5 ml of a 7% perchloric acid solution was added. The contents of the vial were stirred gently and incubated for 30 min. The extract was neutralized with 0.35 ml of 1 M KOH, and the precipitate was spun down in an Eppendorf centrifuge. The radioactivity in 100 μ l of the supernatant was determined in duplicate by scintillation counting. The remaining supernatant was freeze-dried. Dried material was dissolved in a small volume of water and subjected to thin-layer chromatography to see whether other products had been formed. Reference compounds were treated similarly from the perchloric acid precipitation step on.

Determination of intracellular amino acid pools by HPLC analysis of cell extracts. Cultures on CDM were grown to an optical density at 660 nm of 0.5. Cells were separated from the medium by silicon oil centrifugation (20). Eppendorf tubes (1.2-ml volume) were prepared with 0.8 ml of silicon oil (AR 200; Wacker Chemie, Munich, Germany) on top of 0.2 ml of 7% (wt/vol) perchloric acid supplemented with 4.5 mM EDTA. A sample (1 ml) of the culture was layered on top of the silicon oil. The tubes were centrifuged for 6 min in an Eppendorf centrifuge at full speed. Of the extract, 120 μ l was brought to pH 9.5 with 180 μ l of 1 M KOH-1 M KHCO_3 . Debris and salt were pelleted by centrifugation, and 150 μ l of the supernatant was dansylated by adding 75 μ l of dansyl-chloride (1.5 mg/ml in acetonitrile). The reaction was stopped after 30 min of incubation in the dark at room temperature by addition of 2 μ l of 4 mM methylammonium chloride. Dansylated amino acids were analyzed by reversed-phase high-pressure liquid chromatography (HPLC) (μ Bondapak C18 column; Waters [Millipore], Milford, Mass.). A 40-min linear gradient of 5 to 55% (vol/vol) acetonitrile in 30 mM sodium phosphate (pH 6.5) was used to elute the derivatives. Absorption was measured at 254 nm. Peak areas were compared with standard samples and assumed to be proportional to the concentration. The intracellular concentration was calculated by using an intracellular volume of 2.9 μ l/mg of protein (18), and a correction was made for extracellular fluid sedimenting with cells through the silicon oil (20).

Uptake assays. Cells were harvested in the exponential growth phase (optical density at 660 nm of 0.5 to 0.6). Two buffer systems were used which had approximately the same osmolarity as the growth medium. For cells grown on MRS, 200 mM potassium phosphate (pH 6) was used, and for cells grown on CDM, 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6) with and without 0.5 M KCl was used, depending on whether the cells had been grown on CDM with or without KCl. Cells were washed in buffer and depleted of phosphoenolpyruvate and ATP pools by incubation with 5 mM methyl-1-thio- β -D-galactopyranoside for 30 min at 29°C. Starved cells were washed twice in buffer and resuspended. Before the uptake assays, cells were incubated at 29°C and preincubated for 4 min with lactose. Uptake was started by the addition of radiolabeled betaine or proline. In kinetic (initial-rate) experiments with betaine at concentrations above 0.5 μ M, ^{14}C -labeled betaine was used, while for lower concentrations, ^3H -labeled betaine with a higher specific activity was used. Samples (200 μ l) were withdrawn, and uptake was stopped by addition of 2 ml of cold 0.1 M lithium chloride. The suspension was immediately filtered over a cellulose nitrate filter (0.45- μ m pore size; BA85; Schleicher & Schuell), and the filter was washed with 2 ml of

cold lithium chloride. Filters were dried and radioactivity was counted by liquid scintillation.

Selection of a proline auxotrophic mutant. A proline auxotrophic mutant was found in an attempt to isolate osmoregulation mutants which were deficient in betaine uptake. The principle of the procedure was to select mutants which were unable to grow or able to grow only slowly on CDM with betaine and 0.65 M NaCl. The negative selection was facilitated by penicillin suicide enrichment of mutants.

An overnight culture of *L. lactis* subsp. *lactis* ML3 on MRS was harvested and resuspended in 2 mM MgSO_4 to an optical density at 660 nm of 0.1 (approximately 10^8 CFU/ml). Of this suspension, 10 ml was spread in a petri dish and irradiated with UV light. A series of different irradiation time intervals were used. The sample which showed 2% survival of CFU (with a mean chain length of 4 cells, survival of 0.5% of the cells can be calculated) was chosen for further selection. Of this sample, 2 ml was inoculated for 5 h in 50 ml of CDM, pH 7, in the dark to allow segregation of mutations. Subsequently, 50 ml of CDM (pH 7) supplemented with 0.25 mM betaine, 1.6 M NaCl, and 10 mg of penicillin G was added, and the culture was incubated overnight. The culture was harvested, resuspended in 100 mM potassium phosphate (pH 7), and plated on CDM agar (low-osmolarity agar). One thousand colonies were replica plated on low osmotic agar and CDM agar supplemented with 0.25 mM betaine and 0.65 M NaCl (high-osmolarity agar).

The colonies which did not grow on high-osmolarity agar were streaked on low-osmolarity agar. A colony of each mutant was incubated on MRS liquid medium (10 ml) and grown overnight. A droplet of each culture was transferred to 10 ml of fresh medium and grown overnight. This was repeated three times to select stable mutants. The phenotype was checked again on low- and high-osmolarity agar. Two stable mutants were found, of which one appeared to be a proline auxotroph, termed Pro⁻. The other mutant was not defective in betaine transport, and its mutation is still unknown. How this procedure allows the selection of a proline auxotrophic mutant is explained below.

Miscellaneous. The osmolarities of media and buffers were measured by freezing-point depression with an Osmomat 030 (Gonotec, Berlin, Germany).

RESULTS

Stimulation of growth on CDM by betaine at high salt concentrations. The growth of *L. lactis* subsp. *lactis* ML3 was monitored at different growth medium osmolarities. The osmolarity was varied with either KCl or NaCl (Fig. 1). At osmolarities below 1 osmolar (osM), betaine inhibited growth. At higher osmolarities, betaine stimulated growth considerably. The inhibition by betaine at low osmolarities may be due to inhibition of proline uptake by betaine, as will be shown below. Since the endogenous proline-synthesizing capacity of *L. lactis* subsp. *lactis* supports only 40% of the maximum growth rate possible on CDM, this organism is dependent on uptake of proline from the medium to attain the maximum growth rate (22). Growth inhibition was also observed when the medium osmolarity was increased with sorbitol or sucrose. However, in those cases, no significant stimulatory effect by betaine was observed (data not shown).

No attempts were made to study the role of proline as an osmoprotectant, since discrimination between growth stimulation due to osmoprotection and that due to the supply of proline for biosynthesis was not possible.

Intracellular pools of betaine and amino acids in cells grown

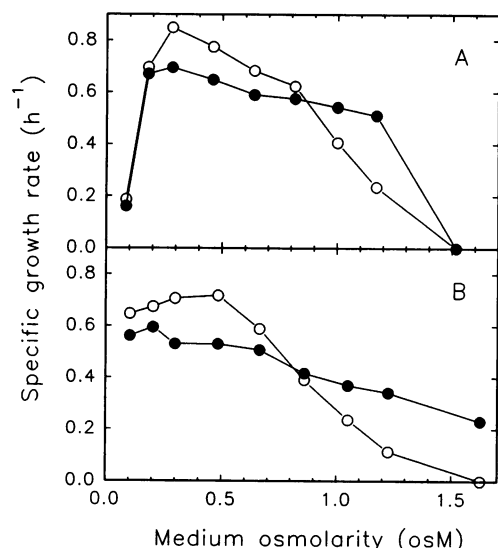


FIG. 1. Effect of betaine and salt on the growth rate of *L. lactis*. The osmolarity of the CDM was varied by addition of KCl (A) or NaCl (B). Open and solid circles represent growth in medium without and with 0.25 mM betaine, respectively. The growth rate was estimated by fitting the optical density at 660 nm time series data to the exponential growth equation.

on CDM at low and high concentrations of KCl. The intracellular concentrations of betaine in growing cells was determined by growing them in the presence of 0.5 mM radiolabeled betaine. In CDM supplemented with 0.5 M KCl and 0.5 mM betaine, the intracellular betaine concentration was 1.1 to 1.2 M. At low medium osmolarity (no KCl added), the intracellular betaine concentration was 0.15 to 0.18 M. Thin-layer chromatography showed that no radiolabeled products other than betaine were present in the cells. Thus, the intracellular betaine pool is scavenged from the medium, and transport seems to be subject to osmoregulation.

Similarly, cells were grown on CDM in the presence of radiolabeled proline (6 mM). At high medium osmolarity (0.5 M KCl added), an intracellular free proline pool (i.e., extractable with perchloric acid) of 240 mM was found, while at low medium osmolarity (no KCl added), the intracellular proline concentration was 8 mM. Again, thin-layer chromatography analysis of cell extracts showed radioactivity migrating only with proline. These results indicate that proline is accumulated from the medium by an active transport system which is subject to osmoregulation. The intracellular concentrations were calculated without taking into account the probably small contribution of endogenously synthesized proline. On CDM without proline, endogenous synthesis sustains an intracellular pool of approximately 0.5 mM, as measured by HPLC amino acid analysis (see also reference 22). Amino acid pool analysis of cells grown on CDM without proline and supplemented with 0.5 M KCl did not indicate enhanced endogenous proline synthesis. Therefore, exogenous proline will be the main source of proline under these circumstances.

The pools of amino acids were determined by HPLC of extracts of cells grown at high and low CDM osmolarities in the presence and absence of betaine. Hardly any difference was found when either 0.5 M KCl (Fig. 2) or 0.5 M NaCl (not shown) was used to increase the osmolarity. The osmolarity of the cytoplasm of cells grown on CDM in the absence of

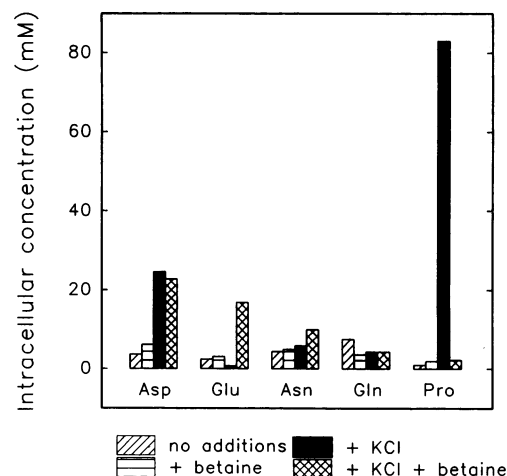


FIG. 2. Pools of amino acids in *L. lactis* cells grown on CDM with the additions indicated (0.5 M KCl and 0.25 mM betaine).

betaine was adjusted by variation of the intracellular pools of proline, aspartate, and glutamate (Fig. 2). Those were the only amino acids that varied significantly in the HPLC traces of dansylated amino acids. However, it cannot be ruled out that other osmolytes were also accumulated at high osmolarities. Aspartate and proline especially were accumulated at high osmolarities (Fig. 2). However, when betaine was present, proline was not accumulated.

These data show that betaine and proline are major osmolytes at high osmolarity. Betaine appears to be the preferred osmolyte.

Characterization of betaine uptake in cells grown on rich broth. The uptake of betaine was assayed in cells grown on different media. The initial uptake rate was found to be similar in cells grown on CDM of low osmolarity and on rich broth (MRS) with and without 0.5 M KCl. This presumably reflects a constitutively expressed betaine uptake system. The presence of this uptake system could, however, not be confirmed for cells grown on CDM of high osmolarity (see below). High rates of betaine uptake were found in the presence of the energy sources lactose and arginine, while in nonenergized cells, no uptake of betaine was observed. Under energized conditions and low osmolarity, accumulation levels of up to approximately 18,000-fold were found at an initial extracellular concentration of 1 μ M betaine. The maximal uptake rate was approximately 4 nmol/min/mg of protein.

Only minor differences in betaine (9 μ M extracellular concentration) uptake rates were observed between cells incubated in buffers of 0.45 osM with different osmolytes (NaCl, KCl, or sucrose) and between cells incubated with different NaCl-adjusted osmolarities (0.35 to 1.6 osM) (data not shown). Apparently, the rate of betaine uptake is not regulated by the osmolarity of the medium.

Betaine did not leak out of cells within 1 h after a 200-fold dilution in medium containing no energy source. Also, no betaine was accumulated when cells had been loaded previously with 3 mM betaine in a medium containing lactose and radiolabeled betaine. Furthermore, counterflow, i.e., exchange of intracellular nonlabeled betaine against a low concentration of extracellular radiolabeled betaine, was not observed under nonenergized conditions in betaine-loaded cells. These observations indicate that the betaine uptake system is kinetically irreversible.

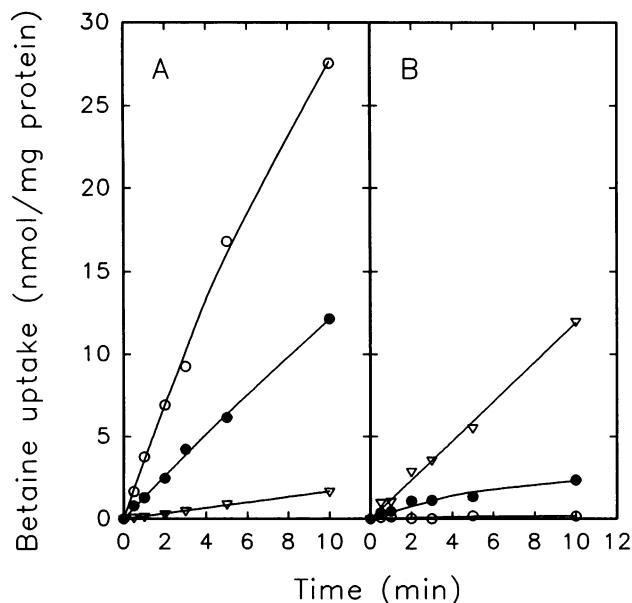


FIG. 3. Effect of buffer pH and addition of ionophores on betaine uptake rate by *L. lactis*. Cells grown on MRS broth were suspended in 200 mM potassium phosphate and energized with 0.5% lactose; 8 μ M 14 C-betaine was added. The buffer pHs were 6 (\circ), 7 (\bullet), and 8 (∇). (A) Coupled cells; (B) cells uncoupled with 2.5 μ M valinomycin and 1 μ M nigericin.

The ionophores valinomycin and nigericin used together abolish the proton and potassium motive forces (14a, 18). Betaine transport in the presence of these ionophores was found (Fig. 3B). This indicates that the transport system is driven by another form of energy than the electrochemical gradients of these ions. The pH had a strong effect on the rate of uptake in both coupled and uncoupled cells (Fig. 3A and B, respectively). However, the effect of these ionophores on the pH sensitivity is peculiar. In coupled cells, a decrease in the medium pH led to an increase in the uptake rate, while in uncoupled cells, the highest uptake rate was observed at high pH. To analyze these effects in more detail, the kinetics of the transport system was investigated. Initial transport rates at different external concentrations of betaine and at different medium pHs are displayed in an Eadie-Hofstee plot (Fig. 4). Transport obeys Michaelis-Menten kinetics. The maximal uptake rate (V_{\max}) decreases with increasing pH, while the Michaelis constant (K_m) remains constant at approximately 1.5 μ M.

In conclusion, *L. lactis* subsp. *lactis* possesses a nonrepressible high-affinity betaine uptake system which is energized independently of the proton motive force.

Characterization of proline transport. In contrast to the betaine transport described above, proline transport is inducible. Cells grown on MRS broth with and without 0.5 M KCl or on CDM of low osmolality displayed only a very low but significant rate of proline transport (Fig. 5). On the other hand, in cells grown on CDM supplemented with 0.5 M KCl, a rate of proline uptake which was 15- to 40-fold higher than in noninduced cells was measured. However, when 5 mM proline was added to MRS with 0.5 M KCl, proline transport was not induced. Clearly, high osmolality is not the only determinant in the induction of proline transport. Probably one or more components of MRS broth repress expression of proline transport.

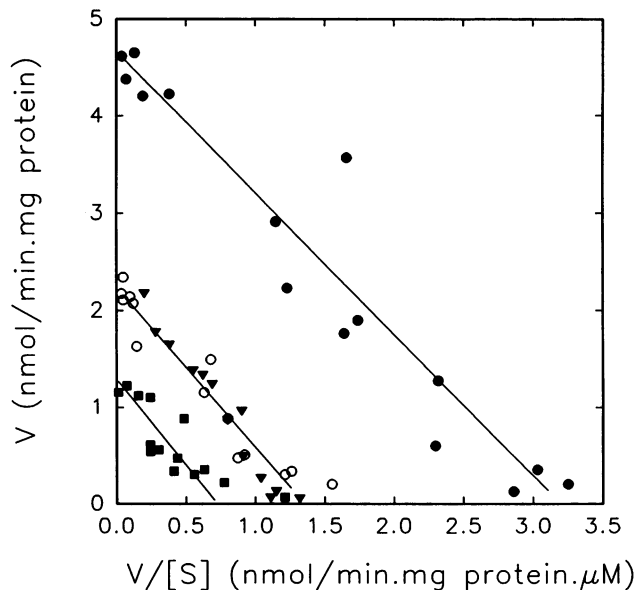


FIG. 4. Effects of pH and ionophores on the kinetics of betaine uptake by *L. lactis*. Cells grown on MRS broth were resuspended in 200 mM potassium phosphate and energized with 0.5% lactose. The initial rates were determined by regression from linear uptake curves with six samples taken at different times (up to 10 min). The pHs were 6 (\bullet), 7 (\circ), and 8 without additions (\blacksquare) or with 2.5 μ M valinomycin and 1 μ M nigericin (∇). The results are presented in an Eadie-Hofstee plot. The betaine concentration range was 0.05 to 115 μ M.

A high rate of proline transport was dependent on energization of cells with lactose (Fig. 6) or arginine (not shown). Proline transport was also stimulated by high medium osmolality. There was hardly any difference in stimulation whether the osmolality was increased with sorbitol, NaCl, or KCl, indicating that the osmolality, and not the particular osmolyte, determined the uptake rate.

The decrease in the intracellular proline pool in CDM

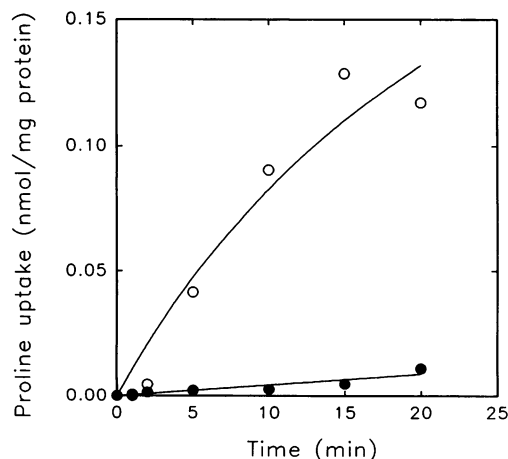


FIG. 5. Effect of growth medium on proline uptake by *L. lactis*. Cells were resuspended in 50 mM MES-0.5 M KCl (pH 6) and energized with 0.5% lactose. After energization, 1 μ M 14 C-proline was added. \circ , cells grown on CDM with 0.5 M KCl; \bullet , cells grown on CDM, MRS, MRS with 5 mM proline, MRS with 0.5 M KCl, or MRS with 0.5 M KCl and 5 mM proline.

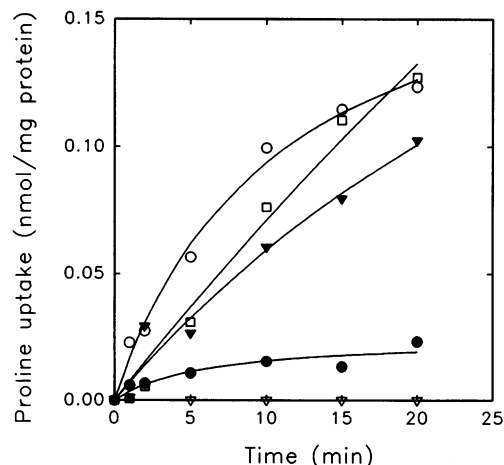


FIG. 6. Energy dependency and osmotic activation of proline transport by *L. lactis*. Proline transport in cells grown on CDM with 0.5 M KCl was determined in 50 mM MES (pH 6) with the following additions: 0.5 M KCl (∇); 0.5% lactose (\bullet); 0.5 M KCl and 0.5% lactose (\circ); 0.5% lactose and 0.5 M NaCl (\square); or 0.5% lactose and 0.82 M sorbitol (\blacktriangledown). Sorbitol at 0.82 M had the same osmolarity as 0.5 M KCl. The ^{14}C -proline concentration was 1 μM .

when betaine was added led to the question of whether betaine influenced proline uptake. Betaine was found to inhibit proline transport very effectively (Fig. 7). The inhibitory constant (K_i) of betaine on proline transport could be estimated from these data by assuming that the proline concentrations used were below the K_m of the transport system for proline (see kinetics, below). This K_i was approximately 50 to 100 μM .

An indication that the proline transport system not only binds but also transports betaine came from the following experiments. Addition of a high concentration of nonradio-labeled betaine or proline to cells which had accumulated radiolabeled proline led to the efflux of this radiolabeled proline, probably through a carrier-mediated exchange of

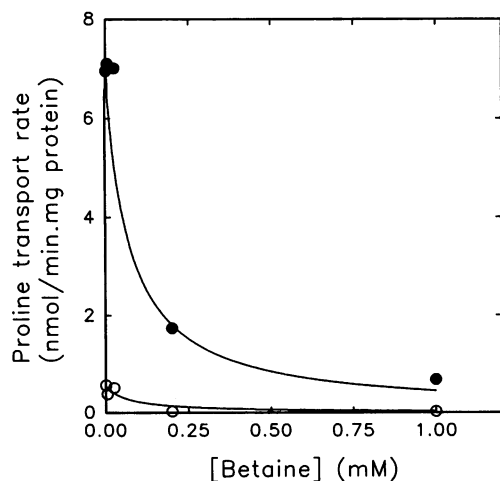


FIG. 7. Inhibition by betaine of proline transport in *L. lactis*. Cells grown on CDM with 0.5 M KCl were resuspended in 50 mM MES-0.5 M KCl (pH 6) and energized with 0.5% lactose in the presence of different concentrations of betaine. After energization, 0.25 mM ^{14}C -proline (\circ) or 4 mM ^{14}C -proline (\bullet) was added.

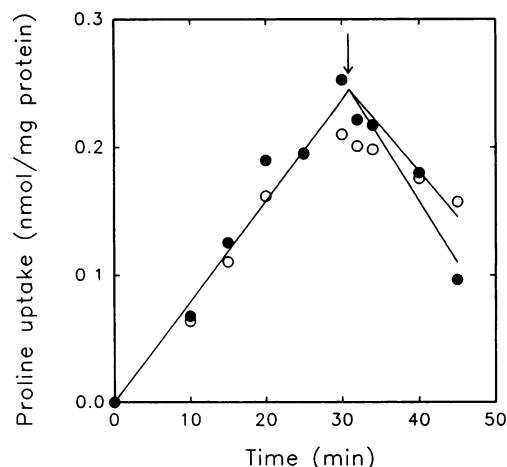


FIG. 8. Effect of extracellular nonradiolabeled proline or betaine on intracellular levels of radiolabeled proline in *L. lactis*. Cells were grown on CDM with 0.5 M KCl, resuspended in 50 mM MES-0.5 M KCl (pH 6), and energized with 0.5% lactose. At time zero, 2 μM ^{14}C -proline was added. After 31 min (arrow) 0.15 mM proline (\circ) or 0.15 mM betaine (\bullet) was added.

intra- and extracellular substrates (Fig. 8). A very high initial rate of betaine uptake was also found in cells grown on CDM with 0.5 M KCl. Within 30 s, a steady state of approximately 8 nmol/mg of protein was reached; the betaine uptake rate was therefore at least fourfold higher than in cells grown on rich broth of low osmolarity. However, this betaine uptake was independent of energization of the cells and may be due to carrier-mediated exchange with intracellular proline.

A kinetic characterization of the transport system with respect to proline uptake indicated that the K_m for proline was above 5 mM (Fig. 9). A good estimation of the kinetic parameters was not possible because at high concentrations of proline, a very low specific radioactivity had to be used, leading to high inaccuracy in the estimated initial rates.

Proline transport was also dependent on the pH of the assay medium (Fig. 10A), although it varied less dramati-

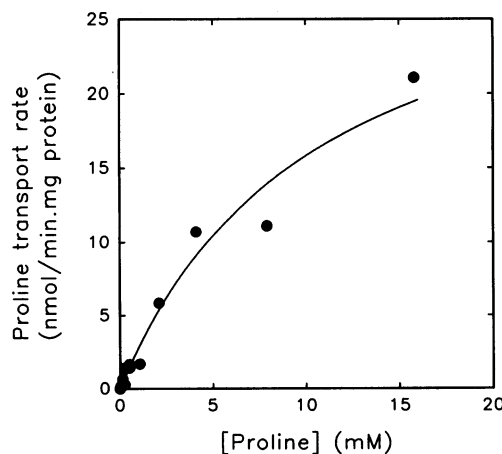


FIG. 9. Kinetics of proline transport in *L. lactis*. Cells grown on CDM with 0.5 M KCl were resuspended in 50 mM MES-0.5 M KCl (pH 6) and energized with 0.5% lactose. The initial rates of proline uptake were estimated from linear uptake curves with four samples taken at different times (up to 4 min).

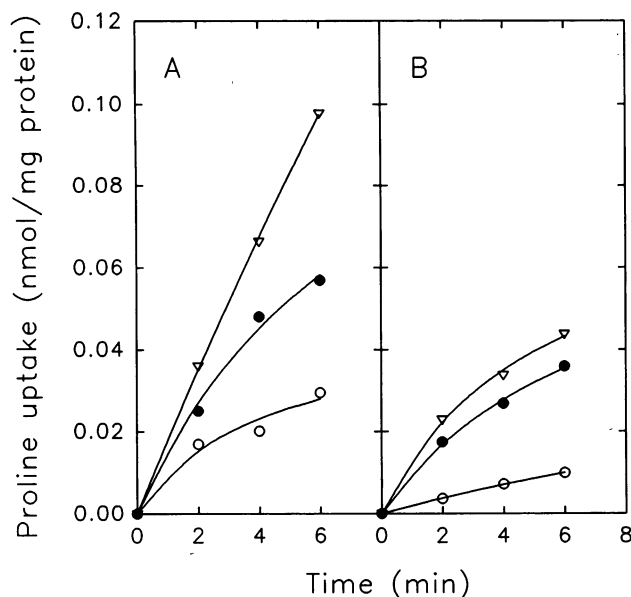


FIG. 10. Effect of buffer pH and addition of ionophores on the rate of proline uptake in *L. lactis*. Cells grown on CDM with 0.5 M KCl were suspended in 25 mM MES–25 mM 1,4-piperazinediethanesulfonic acid (PIPES)–25 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES)–0.5 M KCl and energized with 0.5% lactose. After energization, 1 μ M 14 C-proline was added. The buffer pHs were 6 (○), 7 (●), and 8 (▽). (A) Coupled cells; (B) uncoupled cells. Uncoupling was achieved by addition of 1 μ M valinomycin and 0.5 μ M nigericin.

cally than the high-affinity betaine transport system (compare Fig. 3A). Furthermore, the proline transport rate increased with increasing pH, in contrast to the high-affinity betaine transport. A significant rate of proline uptake was observed in uncoupled cells (Fig. 10B), and this rate increased with increasing pH. Although proline uptake was low under these circumstances, it was far above the equilibration level, which is estimated to be approximately 3 pmol/mg of protein at an external proline concentration of 1 μ M. Uptake was also above the level in nonenergized cells (see Fig. 6), indicating that uptake was not due to carrier-mediated proline-proline exchange. Like the high-affinity betaine transport, proline transport thus occurs independently of the proton motive force.

The observed low rate of proline transport in cells grown on MRS (see Fig. 5) was also inhibited by betaine, although much less effectively. A concentration of 1 mM betaine reduced the uptake rate (proline concentration, 1 μ M) in energized cells by only about 50% (data not shown). This specific inhibition suggests that under these circumstances, proline transport is also mediated by a transport system, although with a low activity, and is not due exclusively to passive diffusion, as was proposed before (22).

Growth characteristics on CDM of a proline auxotrophic mutant. The proline auxotrophic mutant (Pro[−]) was unable to grow on CDM without proline, in contrast to the wild type, which in the absence of proline grows at 40% of the maximal growth rate on CDM (22). The Pro[−] mutant grew on CDM (with proline) supplemented with 0.5 M KCl, and these cells had a proline uptake rate comparable to that of the wild type. However, when 0.5 mM betaine was added to CDM with 0.5 M KCl, the Pro[−] mutant did not grow, in accordance with the selection criterion (see Materials and

Methods). A possible explanation for the lethal effect of betaine under these circumstances may be found in the effective inhibition of proline uptake by betaine because, unlike the wild type, the Pro[−] mutant is absolutely dependent on the uptake of proline for its proline supply. Surprisingly, the Pro[−] mutant was able to grow on CDM of low osmolarity in the presence of 0.5 mM betaine. This observation might be explained by the finding above that proline transport is much less inhibited by betaine under conditions of low osmolarity.

DISCUSSION

The results presented clearly demonstrate that proline and betaine act as compatible solutes in *L. lactis* subsp. *lactis* ML3 and that high intracellular pools of both substances are formed by the activity of specific transport systems. The intracellular osmolyte composition in media in which these osmoprotectants are not present is unknown. The increased aspartate and glutamate concentrations at high osmolarities point to osmoregulation of these pools also.

Cells grown on MRS or low-osmolarity CDM express a possibly constitutive high-affinity betaine transport system. Because the osmotic effects on betaine transport were measured at a 9 μ M betaine extracellular concentration, it cannot be excluded that an osmotically regulated transport system with a low affinity for betaine is also present. The physiological function of a high-affinity, and thus very specific, betaine transport system which is not regulated by the osmolarity of the medium is intriguing. Since betaine is not metabolized by *L. lactis*, a protective function against a stress other than high osmolarity (e.g., low water activity) seems most likely. How the activity of this transport system is regulated is not clear. It was shown that the relationship between pH and transport rate is not unique to these experimental conditions. In uncoupled cells, the relationship was the inverse of that in coupled cells. This suggests that not only pH but also other parameters determine the activity of the uptake system. One of these parameters could be the intracellular concentration of potassium ions. During the generation of a pH gradient across the membrane, potassium ions accumulate rapidly as a consequence of proton extrusion, the buffer capacity of the cytoplasm, and the preservation of electroneutrality (3). At higher extracellular pH, the pH gradient decreases, and the intracellular concentration of potassium ions might also decrease. If betaine transport were activated by intracellular potassium ions, this effect would lead to an apparent activation by low extracellular pH.

The distinctly different osmoregulated low-affinity proline-betaine uptake system in *L. lactis* subsp. *lactis* is reminiscent of the ProU and ProP uptake systems in *Escherichia coli* and *Salmonella typhimurium*. These systems transport proline with a low affinity and betaine with a higher affinity and are activated by high osmolarity at the levels of both transcription and catalytic rate (4, 5, 14). Proline uptake by these systems is strongly inhibited by betaine. Evidence for heterologous betaine-proline exchange catalyzed by the low-affinity proline transport system in *L. lactis* has been presented, and it is an attractive possibility that this system transports betaine with a higher affinity than proline. The extremely rapid energy-independent uptake of betaine in cells in which the proline uptake system had been induced unfortunately prevented a kinetic characterization of betaine uptake in these cells. For other gram-positive bacteria for which osmoregulatory betaine and proline transport systems

have been described, it was claimed that these compounds are transported by separate systems (2, 10, 11, 24). However, evidence was presented recently that an osmoregulatory proline-betaine transport system as well as a high-affinity betaine transport system that is not affected by osmotic pressure are present in *Staphylococcus aureus* (21). This closely resembles the situation described for *L. lactis* subsp. *lactis* in this article.

Proline transport in *L. lactis* subsp. *lactis* was previously thought to be due to diffusion across the membrane (22). The low transport activity in both resting and energized cells and the apparently nonsaturable behavior of proline transport led to this conclusion. However, proline transport is inhibited by betaine both in cells which are not induced and in cells which are induced for osmoregulatory proline transport. Furthermore, there is an indication that the lethality of betaine for the proline auxotrophic mutant grown in CDM of high osmolarity is due to specific inhibition of proline transport. However, since proline transport is stimulated 15- to 40-fold in CDM of high osmolarity (Fig. 5) and inhibited by approximately 90% by 0.5 mM betaine (Fig. 7), one would calculate a net stimulation of proline uptake under these circumstances. This implies either that another compound closely connected with the proline synthesis route limits growth of the Pro⁻ mutant at high betaine concentrations or, more likely, that the transport system has different characteristics in growing cells than under the standard transport assay conditions. Proline diffusion therefore appears not to be sufficient to sustain growth, not even at a high (6 mM) extracellular concentration. These observations strongly suggest that proline transport is catalyzed by specific transport systems under all circumstances.

The energization of both the betaine and betaine-proline transport systems seems to be independent of the proton motive force. This is based on the observation that cells in which the proton motive force has been abolished with valinomycin and nigericin still transport proline and betaine. Uptake of both solutes is dependent on metabolic energy and cannot be explained under all experimental conditions by exchange processes. One possibility is that these transport systems are ATP driven. ATP-driven solute transport has been described for *L. lactis*. These are the glutamate-glutamine uptake system (18, 19) and the BCECF (bis-carboxyethyl-carboxyfluorescein) extrusion system (15). Another possibility is that the betaine and proline-betaine transport systems are driven by a different ion gradient, for example, by a sodium ion gradient.

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